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Expression of SKALP/elafin during wound healing in human skin

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Abstract **Skin-derived antileukoproteinase (SKALP), also known as elafin, is a proteinase inhibitor with specificity for polymorphonuclear leucocyte (PMN)-derived elastase and proteinase-3. SKALP is absent in normal human epidermis, but is strongly induced in inflammatory dermatoses such as psoriasis. SKALP is putatively involved in the regulation of cutaneous inflammation by inhibiting PMN derived proteinases. The aim of this study was to investigate SKALP expression and PMN infiltration during wound healing in human skin. This was examined in healing excisional wounds in normal skin and in impaired healing in various types of chronic venous ulcers. Tissues were analysed using immunohistochemistry and Northern blot analysis. Healing of excisional wounds was studied from day 0 to day 14. An influx of PMN was seen rapidly after wounding and was maximal between day 2 and 4 and then subsided. SKALP was induced within 48 h and was expressed in the suprabasal keratinocytes of the wound edge and the migrating epidermal sheet. SKALP expression was maximal on day 4 and was downregulated at the time of complete reepithelialization (7–14 days). In venous ulcers, PMN were abundant in the wound bed and scarce under the wound edge. SKALP was strongly expressed in the keratinocytes of the wound edge in all types of ulcers studied. In the wound bed, SKALP was not detectable. Our results suggest that SKALP plays a role in the acute, inflammatory phase of wound healing. From the kinetics and topology of SKALP expression we surmise that it negatively regulates PMN infiltration.**

Key words SKALP · Wound healing · Antileukoproteinase · Elastase

Introduction

Proteinase activity is regulated systemically by numerous plasma-derived inhibitors, such as α_1-proteinase inhibitor and α_2-macroglobulin. However, in normal human epidermis only low levels of antiproteinase activity directed against the major polymorphonuclear leucocyte (PMN)-derived proteinases (e.g. elastase, proteinase-3 and cathepsin G) can be detected. Antiproteinase activity is upregulated in inflammatory skin diseases such as psoriasis [5, 18–20] and epidermal tumours [2]. In previous studies we and others have characterized this antiproteinase activity and found it to be an inducible inhibitor of elastase and proteinase 3 [23]. This inhibitor has been named skin-derived antileukoproteinase (SKALP) [18], also known as elafin [23, 24] or elastase-specific inhibitor (ESI) [15]. Cloning of the SKALP cDNA and gene has revealed that the molecule contains several N-terminal transglutaminase substrate motifs, in addition to the antiproteinase domain that was already known [11, 12, 14]. SKALP is a secreted molecule, and we have recently demonstrated its presence in urine and plasma of psoriatic patients [1, 4].

SKALP may be involved in the regulation of cutaneous inflammation by inhibiting PMN-derived proteinases. However, no direct evidence for its function in vivo is available at present. Previous investigations have shown SKALP expression in diseased skin (psoriasis, skin tumours) but have not provided information on the dynamics of SKALP induction or the temporospatial relationship with PMN infiltration at the histological level. Here we studied the kinetics of inflammation and SKALP induction in wound healing of normal skin, which allowed us to examine these processes in more detail. In addition, we studied the spatial relationship between SKALP expression and infiltrating PMN in chronic venous ulcers. Our results demonstrate a coordinated expression of epidermal SKALP and PMN influx in normal wound heal-

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ing. Chronic venous ulcers appear not to progress beyond the inflammatory phase of wound healing, and show the continuous presence of PMN and sustained expression of SKALP by the keratinocytes of the wound margin.

Materials and methods

Chemicals and antisera

A polyclonal antiserum against recombinant SKALP was obtained as described previously [20]. Recombinant SKALP was a kind gift from Dr. N. Russell, ICI Pharmaceuticals, UK.

A monoclonal mouse antibody to human neutrophil elastase, swine-antirabbit immunoglobulin conjugated with horseradish peroxidase (SWARPO) and rabbit-antimouse immunoglobulin conjugated with horseradish peroxidase (RAMPO) were all obtained from Dakopatts, Glostrup, Denmark.

Skin samples

To study impaired wound healing, biopsies were taken under local anaesthesia from the margins of eight chronic venous or mixed venous/arteriolar ulcers, after the patients had given their informed consent and permission from the local medical-ethical committee had been obtained. Patients ranged in age from 29 to 70 years. A rectangle of tissue approximately 10 × 3 mm was excised to include the surrounding intact skin, the ulcer edge and the ulcer bed. The biopsies were taken in clinically different stages of wound healing: two biopsies were taken from an ulcer in the necrotic phase, three in the necrotic/granulating phase and three in the reepithelialization phase. All patients received local wound care and ambulant compression therapy prior to the biopsy. This treatment remained unchanged after the biopsy. All biopsy sites healed within 1–2 weeks, without complications.

To study wound healing in normal skin the following wound model was used in eight subjects with normal skin: after obtaining informed consent four wounds were made under local anaesthesia on the outside of the upper arm on day 0 using a 3-mm punch. The wound depth was approximately 1 mm. On days 2, 4, 7 and 14 standard 4-mm punch biopsies were taken over the previously made wounds.

Immunohistology

Each biopsy was rinsed in phosphate-buffered saline (PBS) before fixing in buffered formalin. After 24 h, tissues were embedded in paraffin wax, sectioned at 5 µm and mounted on 3-aminoalkyltriethoxysilane-coated slides. Sections were deparaffinized, rehydrated and preincubated with either normal swine serum or normal rabbit serum. The sections were then incubated either with polyclonal anti-SKALP/elafin serum or monoclonal anti-elastase. After incubation with peroxidase-conjugated second antibodies, the sections were developed with aminoethylcarbazole as the chromogenic substrate.

Results

PMN infiltration during wound healing

Normal wound healing

Routine H&E staining of biopsies taken over a 14-day period showed the well-known features of normal wound healing in excisional wounds. A dense infiltrate of inflammatory cells was present shortly after injury (24 h), and the wound bed was filled with fibrinous material (not shown). After 4 days all wounds were covered with a scab. Immunohistochemical staining for leucocyte elastase confirmed the presence of large amounts of PMN in the wound bed from day 1 to day 4. The marginal zone of this cellular infiltrate was located just beneath the wound edge. As early as within 2 days reepithelialization had started, and a sheet of ingrowing keratinocytes was visible. Up to 4 days after injury, elastase-positive PMN were prominently present. After 1 week reepithelialization was complete in all wounds. At this time the number of elastase-positive cells had decreased considerably. Only a few scattered PMN remained in the original wound bed. After 2 weeks the keratinocytes had resumed their normal stratification. However, the epidermis still remained acanthotic, and rete ridges were lacking. The cellular infiltrate consisted of mononuclear cells, fibroblasts and endothelial cells. No PMN were present at this time.

Chronic ulcers

On histological examination three zones could be identified within each biopsy: the ulcer bed, the ulcer margin and the surrounding intact skin. The surrounding skin appeared to be normal in most biopsies, except for the acanthotic epidermis. The ulcer margin was covered by epidermis that migrated over, within or under the fibrinous exudate. The ulcer bed varied from a fibrinous exudate to granulating tissue. In all cases many infiltrating cells were visible. In all the ulcer stages studied, similar densities of elastase-positive cells (PMN) were found within the ulcer bed.

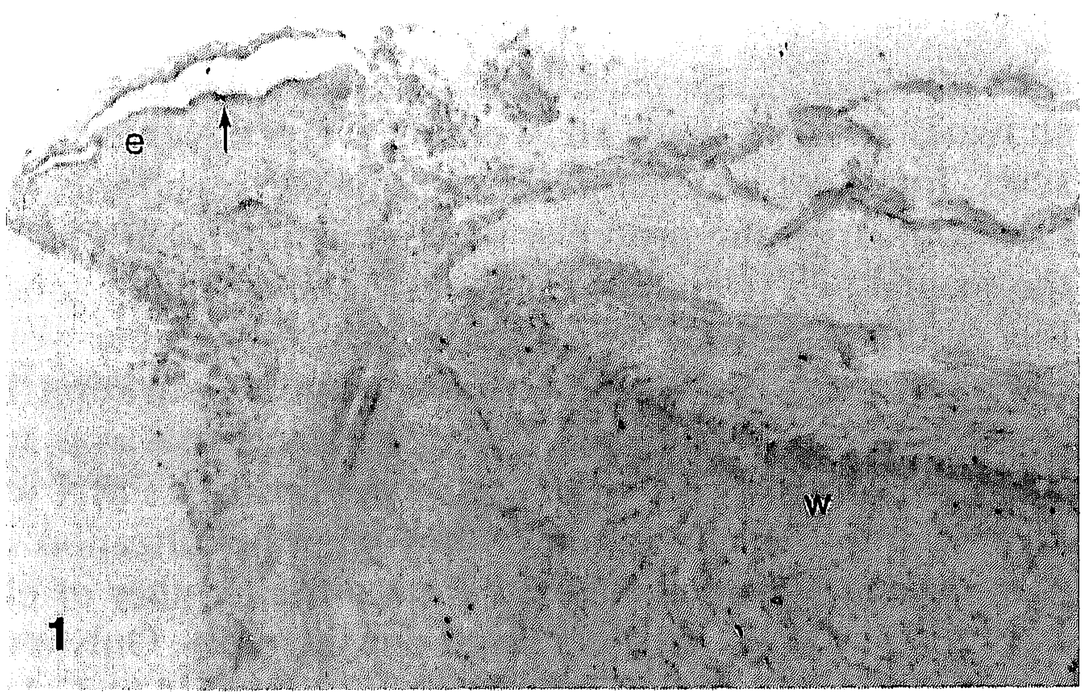
Expression of SKALP during wound healing

Normal wound healing

Within 24 h after wounding, SKALP was induced in the upper layers of the epidermis adjacent to the wound (Figs. 1, 2). On days 2 and 4 expression of SKALP was seen in all cell layers of the migrating sheet of epidermal cells except for the basal layer (Figs. 3, 4, 5). This suprabasal cytoplasmic staining of the neoepidermis decreased towards the intact normal skin. Most of the wounds were already closed 1 week after wounding. Staining for SKALP at this stage was positive up to the original wound edge (Figs. 6, 7). SKALP expression was almost completely downregulated 2 weeks after wounding. In some sections positive staining was still visible in the upper layers of the stratum spinosum.

Chronic ulcers

The expression patterns of SKALP were identical for all three ulcer stages. A positive suprabasal staining was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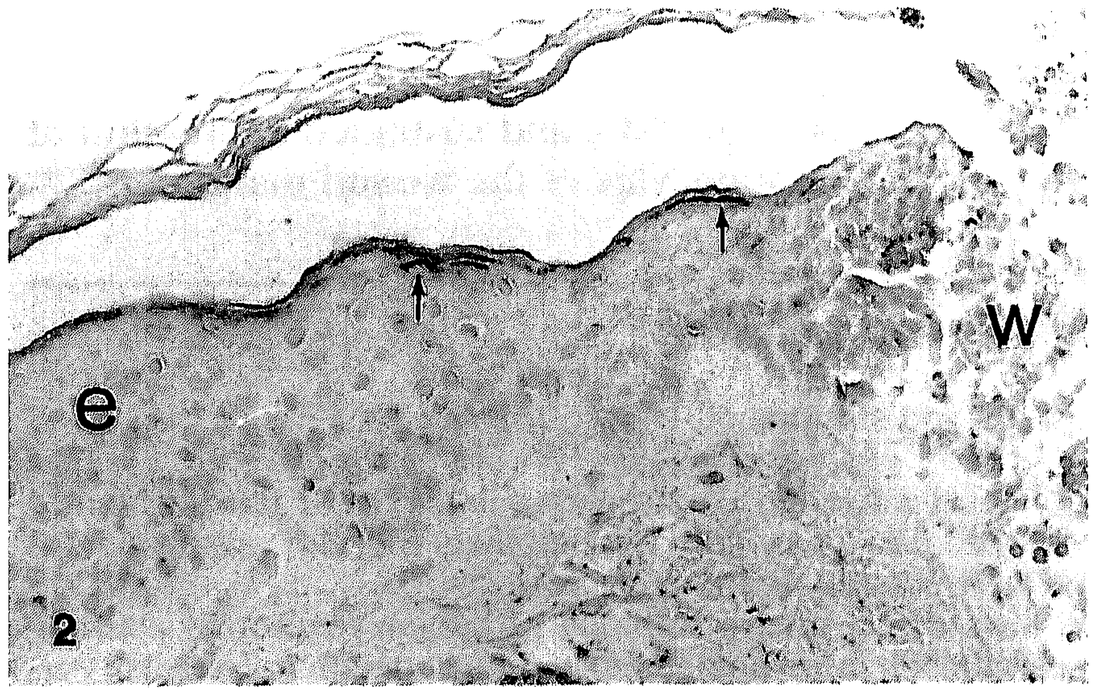
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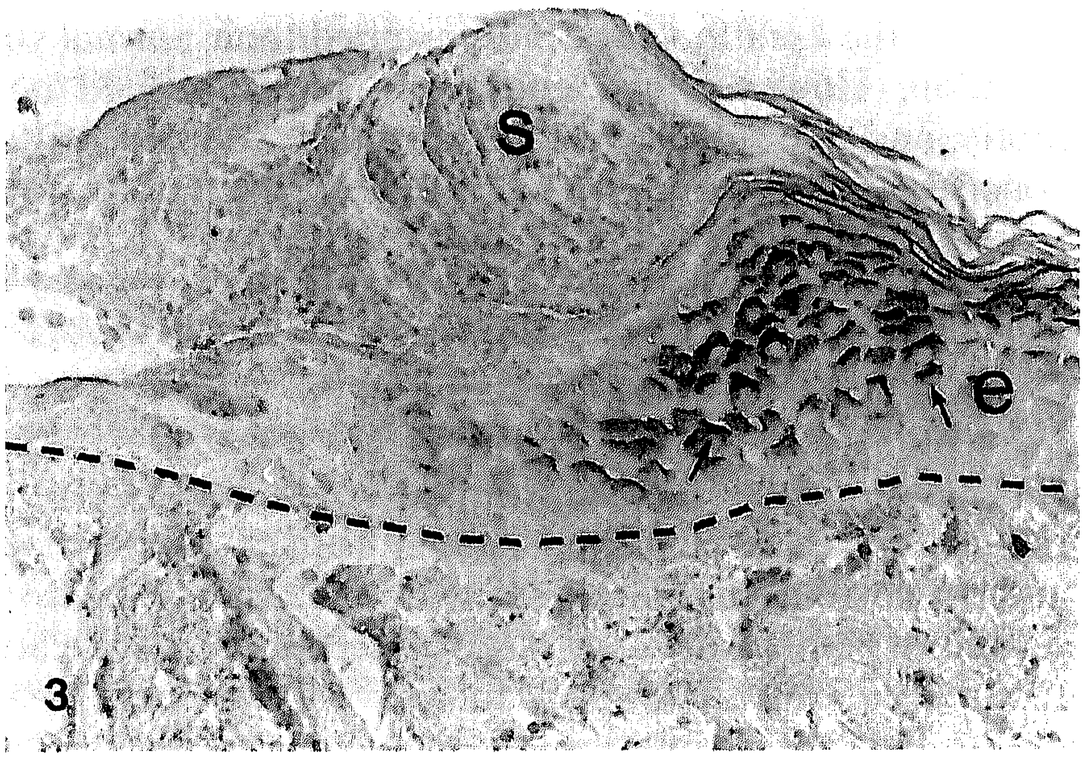
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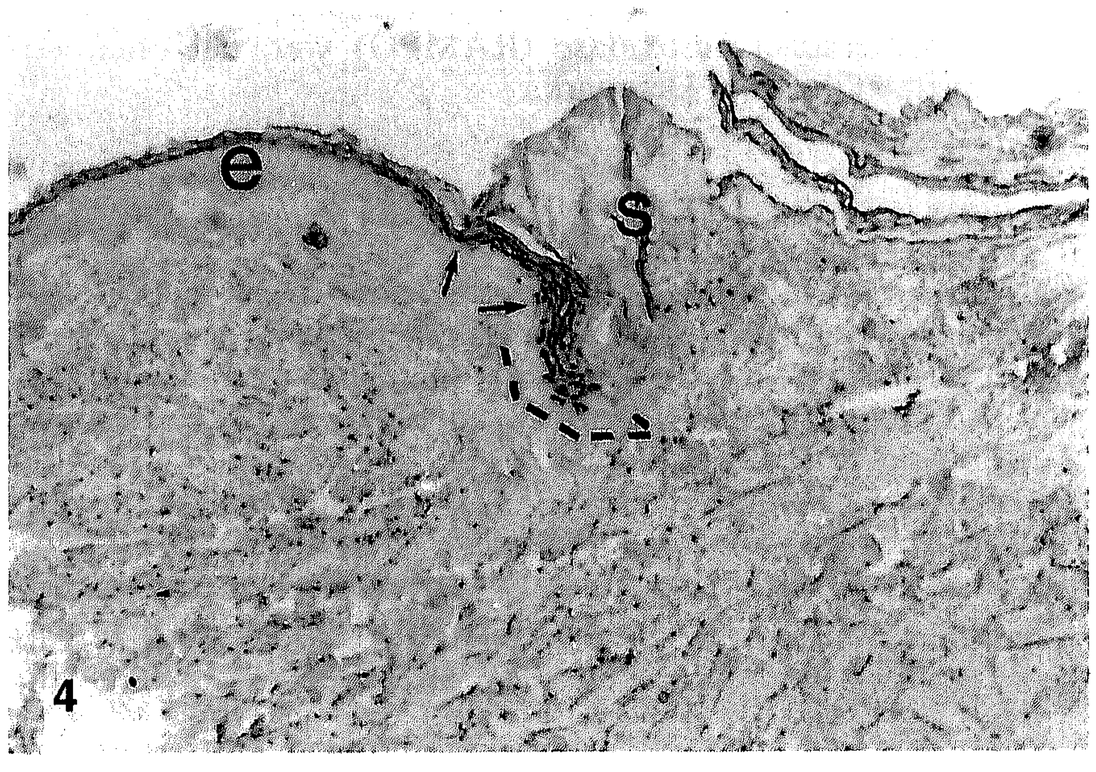
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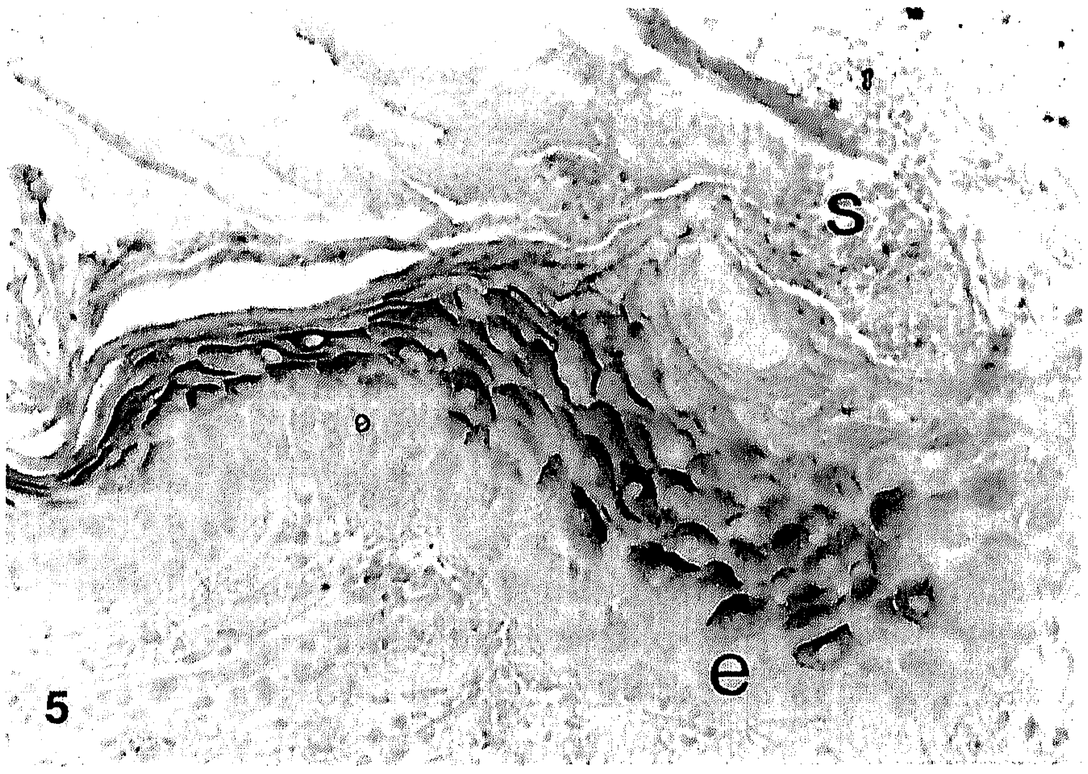
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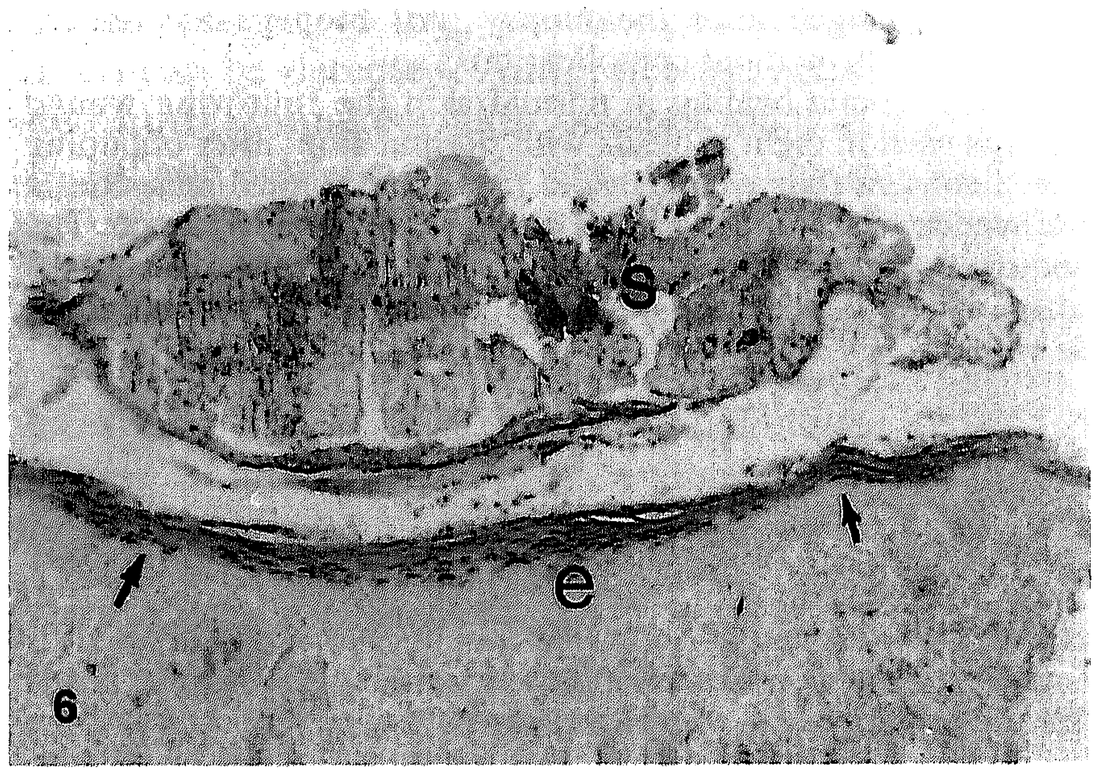
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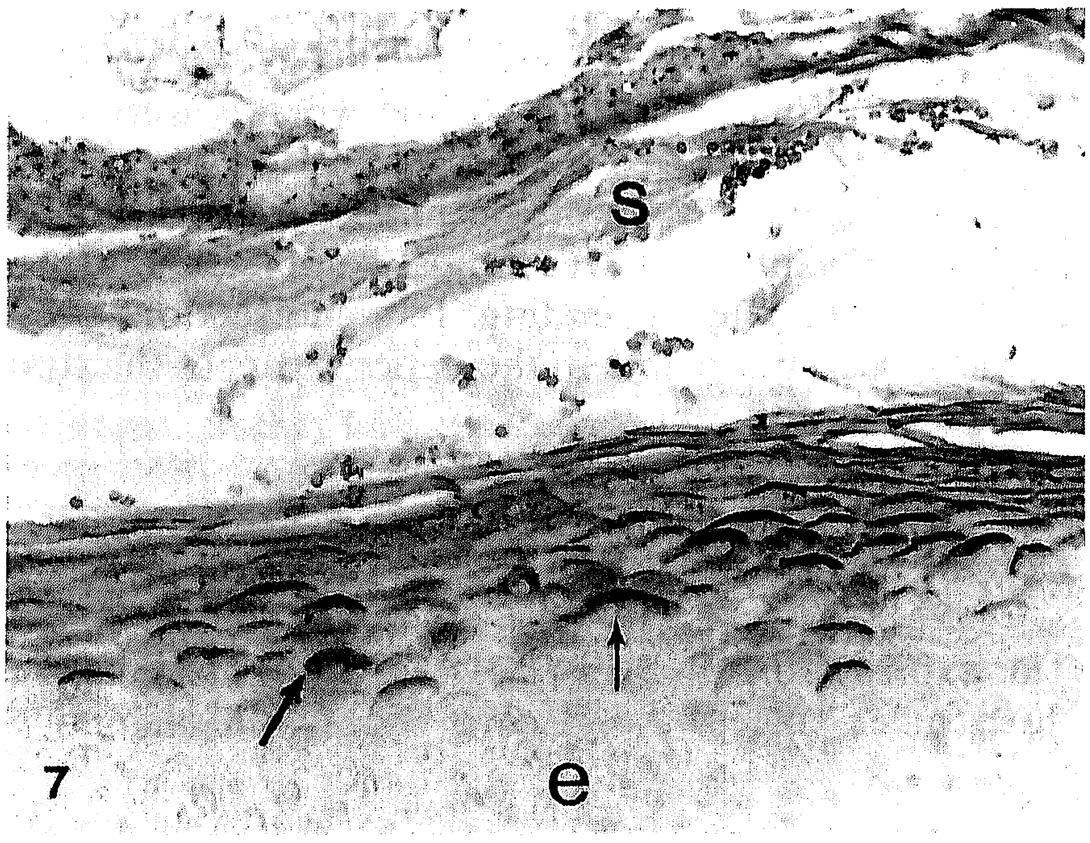
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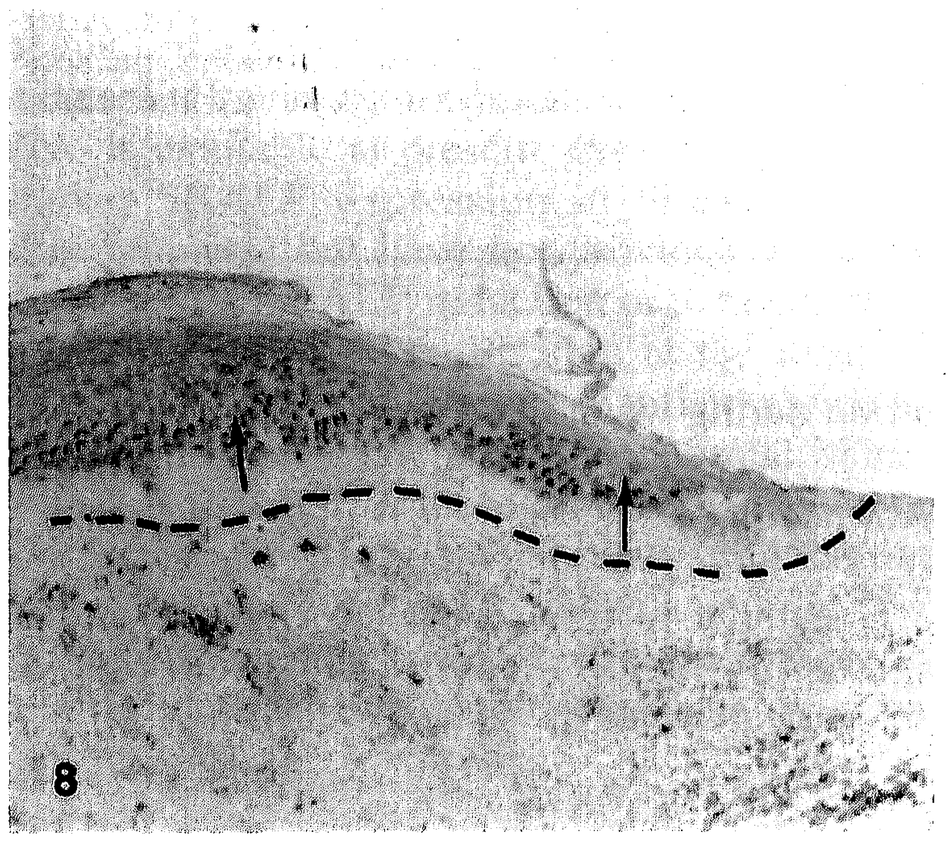
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found in the epidermis at the ulcer margins and in the migrating sheet (Fig. 8). SKALP expression gradually diminished towards normal skin.

Discussion

Wound repair is a complex process comprising inflammation, reepithelialization, matrix synthesis and tissue remodelling. These processes are essential to restore the structural and functional integrity of the damaged tissue. In the early inflammatory phase of wound healing, PMN invade the wound area [7]. The main function of the PMN at the wound site is the elimination of contaminating bacteria via phagocytosis and subsequent enzymatic and oxidative mechanisms [21, 22]. In normal human skin, invasion of PMN is observed as early as a few hours after wounding, decreasing rapidly after several days [13]. In disturbed wound healing, e.g. chronic venous ulcers, PMN remain present in the wound area [7]. It has recently been reported that leucocyte elastase is responsible for extensive fibronectin degradation in burn wounds [9]. Furthermore, in wound fluid from chronic leg ulcers fibronectin is almost completely degraded [10]. Since fibronectin plays an important role in adhesion and migration of epidermal cells [8], degradation of fibronectin and other adhesive matrix components could impair proper reepithelialization. Antiproteinase activity, therefore, could be of crucial importance in establishing an environment which is beneficial for epidermal migration by reducing proteolysis of matrix components.

In previous work we have shown that human epidermal kerytinocytes are capable of producing the elastase/proteinase-3 inhibitor SKALP, in the context of the hyperproliferative/regenerative differentiation programme. This was shown both in vivo in psoriatic lesions and after tape stripping [3, 20]. We report here the kinetics of SKALP induction in full-thickness wounds, and its presence in chronic ulcers. In excisional wounds a PMN influx was seen rapidly after wounding and was maximal between day 2 and day 4, then subsided. SKALP was induced within 48 h and was expressed in the suprabasal keratinocytes of the wound edge. SKALP expression was maximal on day 4 and was downregulated at the time of complete reepithelialization (7–14 days). This sequence of events suggests the SKALP gene expression and the influx of PMN are causally linked.

Sallenave et al. [16] have recently shown that leucocyte elastase is itself capable of inducing SKALP gene expression in pneumocyte cell lines. However, we were unable to induce SKALP expression in cultured keratinocytes under these conditions (Molhuizen et al., unpublished observation). In vitro we have found that serum components can induce SKALP expression at the transcriptional level [3]. Since acute inflammatory processes are usually accompanied by vasodilatation and oedema formation, this could very well be the relevant signal during in vivo wound healing. The observation of the immediate production of SKALP in the newly formed epidermis suggests an important role for SKALP in the early phase of the wound-healing process. SKALP could protect the neoepidermis against damage caused by elastase, thus leading to continuation of reepithelialization. Secretion of SKALP counteracts degradation of adhesive matrix proteins by elastase and proteinase-3. In addition to protection of matrix components, SKALP could also interfere with PMN migration to the inflammatory focus. The observation that PMN accumulated in the wound bed and were absent near the wound margin where SKALP is secreted, supports this suggestion.

As shown immunohistologically, SKALP was expressed at high levels in the wound margins of chronic ulcers. Nevertheless, PMN were continuously present and reepithelialization was severely impaired. Chronic ulcers appeared unable to progress through the normal stages of wound healing. A chronic ulcer represents as unstable equilibirum between continuous inflammatory stimulation (influx of PMN) and a frustrated restorative response (reepithelialization, SKALP expression). The pathogenetic mechanisms for ulcer formation are largely unknown. Active inflammation and excessive secretion of PMN-derived proteinases could saturate the local protective mechanisms, as suggested by the massive breakdown of matrix proteins in ulcer wound fluid [9]. These findings may provide a rational basis for the use of proteinase inhibitors as a therapeutic modality in chronic ulcers.

◀ **Fig. 1** Histology of a 1-day-old wound in the upper arm of a healthy volunteer with immunoperoxidase staining for SKALP. Note the first discrete signs of SKALP expression *(arrow)* in the upper layers of the epidermis *(e)* adjacent to the wound *(w)* (× 45)

Fig. 2 Histology of a 1-day-old wound in the upper arm of a healthy volunteer with an immunoperoxidase staining for SKALP. Detail of Fig. 1. The first signs of SKALP expression *(arrows)* in the upper layers of the epidermis *(e)* adjacent to the wound *(w)*. (× 180)

Fig. 3 Histology of a 2-day-old wound in the upper arm of a healthy volunteer. Note the marked immunohistochemical staining for SKALP *(arrows)* in all suprabasal cell layers of the epidermis *(e)* migrating under the scab *(s)*. (× 180)

Fig. 4 Histology of a 4-day-old wound in the upper arm of a healthy volunteer. There is a strong suprabasal immunoperoxidase staining for SKALP *(arrows)* in the epidermis *(e)* migrating under the scab *(s)*. (× 45)

Fig. 5 Histology of a 4-day-old wound in the upper arm of a healthy volunteer. Detail of Fig. 4. There is a strong suprabasal immunoperoxidase staining for SKALP in all suprabasal cell layers of the epithelium *(e)* migrating under the scab *(s)*. (× 180)

Fig. 6 Histology of a 7-day-old wound in the upper arm of a healthy volunteer. The wound is already closed underneath the scab *(s)*. There is still a suprabasal staining present for SKALP *(arrows)*, mainly in the upper layers of the epidermis *(e)*. (× 45)

Fig. 7 Histology of a 7-day-old wound in the upper arm of a healthy volunteer. Detail of Fig. 6. Note the suprabasal immunoperoxidase staining for SKALP *(arrows)* in the upper layers of the epidermis *(e)* under the scab *(s)*. (× 180)

Fig. 8 Histology of a chronic leg ulcer. The sheet of ingrowing epidermis is indicated by a *dotted line*. Note the suprabasal, epidermal, cytoplasmic expression of SKALP *(arrows)*. (× 45)

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